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Autosomal Dominant Polycystic Kidney Disease (ADPKD) in an Italian Family Carrying a Novel Nonsense Mutation and Two Missense Changes in Exons 44 and 45 of the PKD1 Gene

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Sixty-seven Italian patients with autosomal dominant polycystic kidney disease (ADPKD) were screened for mutations in the 3′ unique region of the PKD1 gene, using heteroduplex DNA analysis. Novel aberrant bands were detected in 3 patients from the same family. DNA sequencing showed a C to T transition in exon 44 (C12269T), resulting in a premature stop codon (R4020X), predicted to impair the synthesis of the putative intracytoplasmic C-terminus tail of the PKD1 protein, polycystin. The mutation also generates a novel DdeI restriction site, and the abnormal restriction pattern was observed both on genomic DNA and on cDNA from the affected relatives, indicating that this is indeed the pathogenetic molecular lesion. Reverse transcriptase-polymerase chain reaction (RT-PCR) performed on lymphocyte mRNA showed that the mutant transcript is normally present and stable. No aberrantly spliced mRNAs were detected. Interestingly, the mutant PKD1 chromosome in this family also bears two missense mutations downstream (A12341G and C12384T), not found in the other ADPKD families studied.

KEY WORDS: **ADPKD, heteroduplex analysis, mutation detection, nonsense mutations, PCR, PKD1 gene**

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common potentially fatal single-gene diseases, and the most common genetic cause of renal failure in adults. It is characterized by bilateral progressive cystic dilatation of the renal tubules, leading to end stage renal disease (ESRD) in adult life. Hepatic cysts, cerebral aneurysms, and cardiac valve abnormalities may also be found [Gabow, 1993]. ADPKD is heterogeneous, with at least 3 genes involved: PKD1 at 16p13.3, which accounts for most ADPKD families [Peters et al., 1992], PKD2 at 4q13–23 [Kimberling et al., 1993; Peters et al., 1993], and PKD3, still unmapped [de Almeida et al., 1995; Daoust et al., 1995]. Heterogeneity of ADPKD may in part explain its clinical variability, PKD1 being usually associated with a more severe course and earlier onset, as compared to PKD2 [Ravine et al., 1992]. However, intrafamilial variability has also been documented [Gabow, 1993].

The PKD1 gene was identified recently and characterized [European PKD Consortium, 1994; American PKD1 Consortium, 1995; International PKD Consortium, 1995], and predictions were made about the structure and functions of its product, polycystin [Hughes et al., 1995]. However, mutation detection has been complicated by the reiteration of over 3/4 of the PKD1 gene proximally on the same chromosome [European PKD Consortium, 1994]. These repeated PKD1-like sequences are transcriptionally active. The precise role of the PKD1 protein is still unknown, even though it might be involved in interactions between cells or with the extracellular matrix. Only a few mutations have been described so far in the PKD1 gene, including

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deletions [European PKD Consortium, 1994; Brook-Carter et al., 1994], splice mutations [European PKD Consortium, 1994; Peral et al., 1995], and a nonsense mutation [Turco et al., 1995]. Here we report the identification of the second premature termination codon in another ADPKD Italian family.

MATERIALS AND METHODS

Family Description

Part of the other Italian ADPKD families in which linkage and mutation studies were performed have been described previously [Turco et al., 1991, 1995]. The family described here (VR4137T) was first referred for genetic counseling in 1988, for the presence of ADPKD in 3 generations (Fig. 1). Linkage analysis was performed with markers on 16p [D16S85 (probe 3′HVR), and D16S80 (probe 24.1)]. At the time of counseling 5 affected subjects (I-2, II-1, II-2, III-1, and III-4 in Fig. 1) were deceased. I-2 died of chronic renal failure in old age. II-1, and II-2 died of ESRD. II-5 was born in 1933, and at the age of 50, during routine controls, a diagnosis of polycystic kidney and liver was made. A treatment with protein restriction was started but one year later dialysis was required. At age 52 a first episode of subarachnoid hemorrhage took place, then hypertension, hematuria, urinary tract infections, thrombosis developed, she died of sepsis. Her daughter (III-8), born in 1954, was diagnosed at 17 years as bearer of polycystic kidneys. No liver cysts were detected. She has been symptom-free up to 39 years, when she was admitted to hospital for hyperthermia and hematuria. At that time urea clearance was 40 ml/min, and creatinine clearance was 40 ml/min. She is now in good general condition. Individual III-5 was born in 1939. His mother (II-2) died at age 58 of ESRD. The first symptoms were macrohematuria and kidney stones at the age of 30. At 43 years, following an episode of aphasia, a CT scan showed a temporal hemorrhage. At 47 years he was admitted to the hospital for a transient ischemic attack. Urea clearance was 35 ml/min, and creatinine clearance was 20 ml/min. At 49 years a diagnosis of polycystic kidney and liver was made by ultrasonography. The patient is now undergoing hemodialysis.

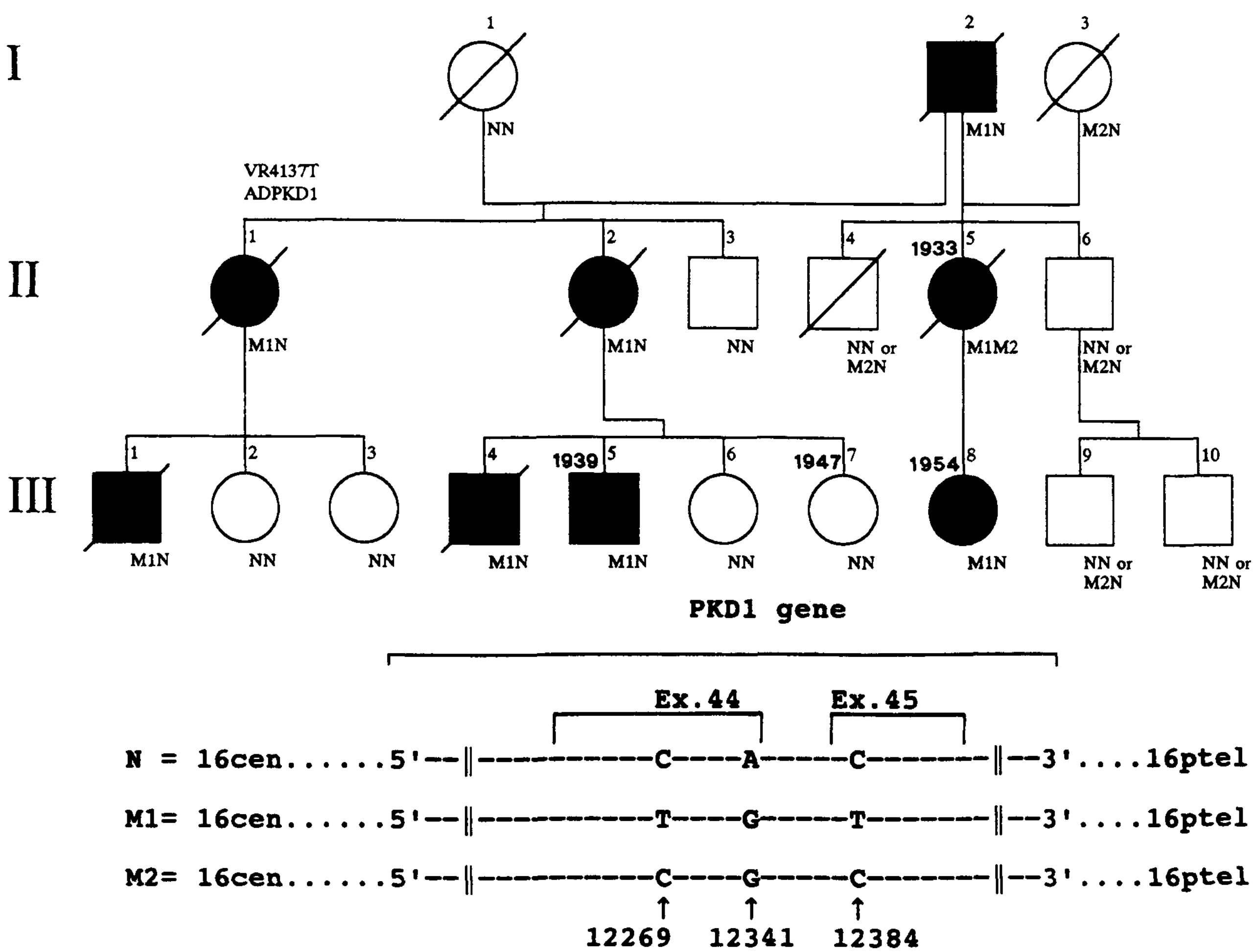

Fig. 1. **Top:** Pedigree of the ADPKD family 4137T. For the 4 individuals analyzed, II-5, III-5, III-7, and III-8, the observed haplotypes and dates of birth are indicated. For the other members the inferred haplotypes are shown. For individuals II-4, II-6, III-9, and III-10, not examined, the possibility of the M2N genotype has also been considered. **Bottom:** Schematic representation of the normal and mutant haplotypes. N, normal PKD1 allele; M1, disease-causing allele, which carries the following three mutations, from the 5′ end: C12269T (R4020X), A12341G (I4044V), and C12384T (A4058V); M2, variant PKD1 allele carrying only the A12341G (I4044V) change.

Amplification and Heteroduplex Analysis of Genomic DNA

DNA was prepared from peripheral blood following standard procedures [Old, 1986]. We used the PCR primers 3A3C1–RS2 for genomic amplification (457 bp), and RS1–RS2 for RT-PCR (240 bp) [European PKD Consortium, 1994; Turco et al., 1995]. PCR conditions were as follows: 5 min at 94°C, then 30 cycles at: 30 sec at 94°C, 30 sec at 61°C, 30 sec at 72°C, followed by 10 min at 72°C. The total PCR volume was 50 μl, using 0.2 Units SuperTaq (HT Biotechnology, UK), and 1.5 mM $MgCl_2$. Heteroduplex analysis was performed using Hydrolink Mutation Detection Enhancement (MDE) gels (AT Biochem, Malvern, PA), with the addition of 15% (w/v) urea, as described [Peissel et al., 1994; Rossetti et al., 1995]. Briefly, 20 μl of the PCR product were denatured by heating at 95°C for 5 min, cooled down at 37°C for 2 hours and loaded. Gels were typically 40 cm long, 0.8 mm thick, were run at 10 V/cm, stained with ethidium bromide and photographed under UV light [Rossetti et al., 1995].

RNA Extraction and RT-PCR

Total RNA was extracted from freshly Ficoll-Paque (Pharmacia, Uppsala, Sweden) collected peripheral lymphocytes, using the RNeasy Total Kit (QIAGEN, Chatsworth, CA). cDNA was synthesized from 1.5 μg of total RNA in a volume of 50 μl. RT-PCR was performed using poly(T) Oligo d(T)16,Cetus. RS1–RS2 cDNA-PCR product of 240 bp was purified by agarose gel using QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA), and digested subsequently.

Automated Sequencing and Restriction Analysis

The PCR products were cloned in pCR TM SK(+) plasmid vector (Stratagene, La Jolla, CA), in the presence of SrfI. Automated sequencing was performed on an ALF DNA Sequencer (Pharmacia LKB, Uppsala, Sweden), using the Autoread Sequencing Kit (Pharmacia, Uppsala, Sweden), following the manufacturer's instructions. Restriction analysis was performed according to the manufacturer's instructions (Promega, Madison, WI). DdeI recognizes the following sequence: CTNAG.

RESULTS

Segregation analysis with PKD1 markers in family 4137T showed possible linkage to PKD1, as indicated by slightly positive lod scores. PKD2-linked markers showed no linkage to this gene (not shown). PKD1 mutation screening was performed by heteroduplex DNA analysis in 67 Italian ADPKD patients from 50 unrelated families (50 probands) from Northern Italy, using PCR primers 3A3C1–RS2 from the unique 3′ end region of the PKD1 gene [European PKD Consortium, 1994; Turco et al., 1995]. Local gene deletions or rearrangements were not detected because the expected 457 bp genomic fragment was found in all probands. Analysis of subjects II-5 and III-8 of family 4137T (Fig. 1) showed the presence of a slower migrating band, as shown in Figure 2, left panel. The different migration of the heteroduplexes in the 2 samples is probably due to different mismatches in the 2 patients carrying different PKD1 haplotypes (M1,M2 and M1,N; Fig. 1, bottom panel).

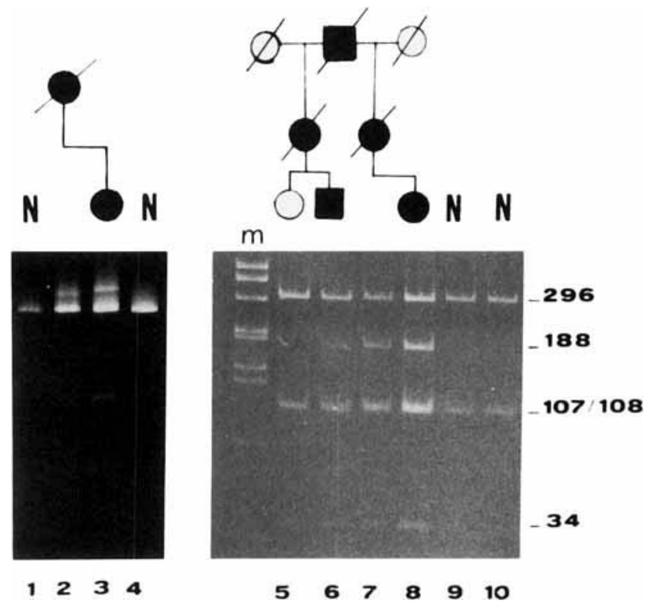

Fig. 2. Genomic DNA analysis. **Left:** Heteroduplex analysis. **Lanes 1** and **4:** Normal unrelated controls (N). **Lane 2:** Patient II-5. **Lane 3:** Patient III-8. A heteroduplex band is present in ADPKD affected individuals. Primer pairs were 3A3C1–RS2 (see Materials and Methods). **Right:** DdeI restriction analysis on amplified genomic DNAs. **Lane 5:** Healthy family member III-7. **Lanes 6–8:** Patients III-5, II-5, and III-8, respectively. **Lanes 9** and **10:** Unrelated controls (N). The C12269T mutation creates a novel restriction site, which gives rise to the additional fragment of 188 bp (clearly visible) and 108 bp (overlapping with the normal 107 bp fragment). m, molecular weight markers (1kb Ladder, BRL, Bethesda, MD). Fragment sizes are shown on the right side in bp. Six percent polyacrylamide gel. Primers used were: 3A3C1–RS2.

We observed cosegregation between heteroduplex bands and disease status in all available affected relatives. To explain the different electrophoretic mobility of heteroduplexes in the two patients, DNA was subsequently cloned and automatically sequenced. A C to T transition was found in both patients II-5 and III-8 at nucleotide position 12269 of the published cDNA sequence [Hughes et al., 1995], changing a CGA codon for arginine to a TGA stop codon (R4020X; not shown). In addition, a homozygous missense transition (A12341G) was detected in exon 44 in patient II-5 (haplotype M1M2 in Fig. 1) and another C to T missense transition was found at nucleotide position 12384, in exon 45, in both II-5 and III-8 (C12384T), in coupling with the affected allele (M1 haplotype in Fig. 1). Both mutations are downstream of the pathogenetic stop codon and change an isoleucine and an alanine, respectively, into a valine (I4044V and A4058V). These missense mutations were not found in 49 different PKD chromosomes (out of the 50 unrelated probands), nor in 70 normal chromosomes. Mutation C12269T creates a novel restriction site for the enzyme DdeI (CCGAG to CTGAG). Restriction analysis performed on genomic DNA showed the presence of both the normal (296+107+34 bp) and mutant (188+108+107+34 bp) PKD1 allele in

the 3 patients II-5, III-5, and III-8 (Fig. 2, right panel). The stability of PKD1 transcript was investigated by lymphocyte cDNA heteroduplex analysis, which showed heteroduplex bands in affected but not in unaffected members (Fig. 3A), thus indicating that the mutant PKD1 allele is transcribed. RNA transcript stability was also assessed, taking advantage of the DdeI restriction site created by the mutation, to distinguish the normal from the mutant alleles in peripheral blood lymphocytes (Fig. 3B). RT-PCR was performed using the primer set RS1–RS2 (see Materials and Methods). The expected fragment of 240 bp was obtained in 2 affected members of the family, in a healthy relative, and in a normal control. Following digestion of the amplified cDNAs with DdeI, we obtained the cleavage of the 240 bp fragment in 2 fragments of 154 and 86 bp, corresponding to the mutant allele in the patients, the normal one remaining undigested. The intensity of the 2 species of transcript was similar, indicating that the mutant transcript is stable (Fig. 3B). The position of the observed mutations along the polycystin molecule is shown schematically in Figure 4.

DISCUSSION

During the search for ADPKD1-causing defects in Italian families, we identified a novel nonsense C to T mutation in exon 44 of the PKD1 gene (C12269T), segregating with the disease. This is the second termination mutation described by us in 50 unrelated Italian ADPKD probands studied thus far, occurring in the same exon [Turco et al., 1995], suggesting that this part of the gene is functionally critical. Mutation C12269T occurs at a CpG site, consistent with the high mutation rate of this dinucleotide, due to methylation-mediated deamination of 5-methylcytosine [Cooper and Krawczak, 1993]. Mutation C12269T is expected to cause the loss of the last 382 C-terminal aminoacids of polycystin, including the whole intracytoplasmic C-tail terminus, the last 2 transmembrane domains, and 3 potential phosphorylation sites (Fig. 4). The intracytoplasmic C-tail domain of the protein, approximately 230 amino acids long, encoded by the unique 3′ end of the PKD1 gene, is probably involved in the transduction of extracellular signals [Hughes et al., 1995]. Our findings show that the mutant PKD1 allele is efficiently transcribed, at least in lymphocytes, and that the loss of this portion of the PKD1 protein is sufficient to generate the disease. An interesting and unexpected finding of this study was the detection of 2 missense mutations (A12341G and C12384T), downstream the nonsense mutation, occurring in the last 2 transmembrane domains (TM10 and 11) of the protein, respectively (Fig. 4). A possible causative role for these missense mutations would be suggested by the failure to observe these changes in around 120 chromosomes analyzed so far (49 affected and 70 unaffected chromosomes), nor in other ADPKD populations. The presence of these 2 sequence variants in the same family has no clear explanations. It might be a chance simultaneous occurrence of rare allelic variants.

The identification of additional mutations in PKD1 patients will lead to a better understanding of the precise normal role(s) of polycystin.

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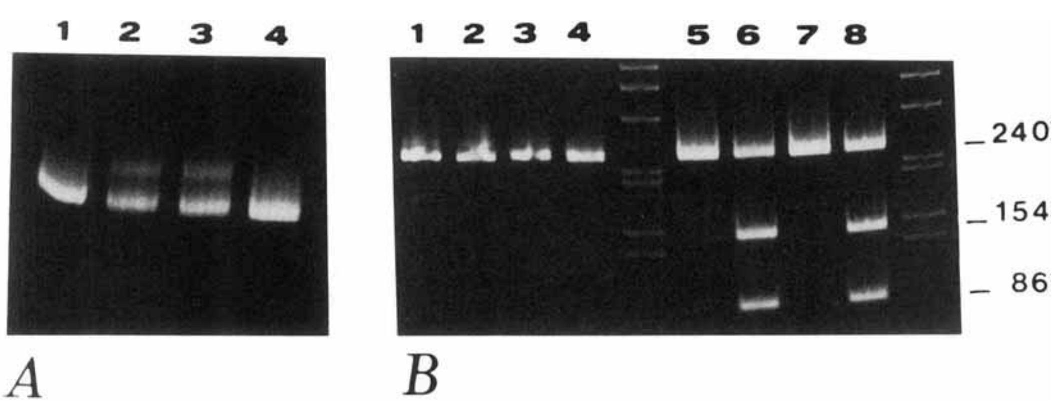

Fig. 3. cDNA analysis. **A:** Heteroduplex pattern of RT-PCR amplified cDNAs obtained from peripheral lymphocyte mRNA. **Lane 1:** Normal unrelated control. **Lanes 2** and **3:** Affected members III-5 and III-8, respectively. **Lane 4:** Unaffected member III-7. Note that the 2 patients share the same PKD1 haplotypes (M1,N) and no difference in heteroduplex migration pattern is observed. Fresh blood from II-5 (deceased) was not available for RNA studies. Primers were: RS1–RS2 (see Materials and Methods). **B:** DdeI restriction analysis performed on cDNA. **Lanes 1–4:** Undigested samples (normal control, III-5, III-7, and III-8, respectively). **Lanes 5–8:** Digested samples (normal control, III-5, III-7, and III-8). The wild-type 240 bp cDNA fragment is not cleaved (lanes 5 and 7). The mutant fragment is cleaved into two aberrant bands of 154 and 86 bp. Six percent polyacrylamide gel. Primers were: RS1–RS2.

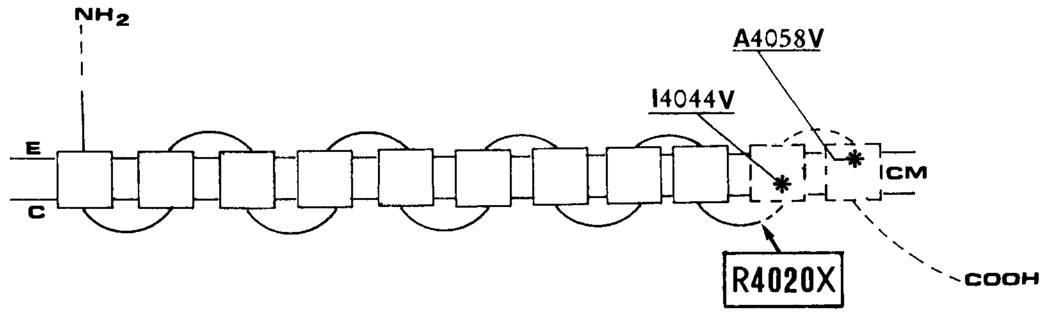

Fig. 4. Schematic drawing of the C-terminal region of the PKD1 product, polycystin, showing the positions of the three mutations described. E, extracellular space; C, cytoplasm; CM, cell membrane. Squares represent the 11 putative transmembrane (TM) domains. The interrupted line at the C-terminus indicates the part of the protein expected to be missing due to the R4020X mutation (boxed). Asterisks within the TM10 and TM11 refer to the positions of the two missense mutations. Drawing not to scale. The polycystin model is modified after Hughes et al. [1995].

NOTE ADDED IN PROOF

Other novel PKD1 mutations have been quite recently identified in ADPKD patients, including two deletions, a single base insertion, two premature terminations, and a missense mutation [Peral B, San Millan JL, Ong ACM, Gamble V, Ward CJ, Strong C, Harris PC (1996): Screening the 3′ region of the polycystic kidney disease 1 (PKD1) gene reveals six novel mutations. Am J Hum Genet 58:86–96].

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